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EFFECT OF ENDOGENOUS AND EXOGENOUS GHRELIN ON GASTROINTESTINAL FUNCTION IN RAT AND HUMAN

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”Whether you believe you can do a thing or not, you are right”

Henry Ford

ABSTRACT

Background

Several peptides derived from the gastrointestinal tract (GI) have been shown to have profound effects on GI motility, food intake and metabolism. Two such peptides, derived from the same prohormone, are the peptides ghrelin and obestatin, discovered in 1999 and 2005, respectively. The ghrelin receptor (GHS-R) is distributed in a large number of central and peripheral tissues, such as, the GI tract, pancreas, kidney, fat, skeletal muscle and several parts of the brain. The obestatin receptor still remains unknown. Two major molecular forms of ghrelin, acyl and des-acyl ghrelin exist. They regulate GI motility peripherally in the local enteric nervous system, but also by activating hypothalamic peptides via the vagal nerve or the bloodstream by crossing the blood-brain barrier (BBB). Ghrelin is involved in a variety of metabolic functions by affecting the glucose homeostasis, fat metabolism, appetite and meal initiation. The role of obestatin and its interaction with ghrelin is still uncertain.

Aims

The main aims of this thesis were to investigate the role of obestatin and ghrelin in GI motility *in vitro* and *in vivo* in rodents and man.

Material and methods

Gastric emptying and small bowel motility were studied *in vivo* in rats with implanted gastric catheter and intestinal electrodes. Gastric emptying, oro-caecal transit, colonic transit and gut peptides were assessed in man. Ghrelin, obestatin or saline was infused in rats. Ghrelin or saline was infused in normal humans for 6 hours. *In vitro* studies with human GI tissue were also performed. Gastric emptying was studied in patients randomised to 3 hours saline or ghrelin infusion, before and day 2 after open colo-rectal surgery. Gastric emptying and changes in serum levels of GI peptide hormones were studied in obese subjects subjected to a liquid meal with acetaminophen before, 3 days, 2 months and 1 year after Roux-en-Y gastric bypass (RYGB) surgery.

Results

Whereas ghrelin stimulated GI motility, obestatin did not affect motility *in vitro* or *in vivo* in rats. Ghrelin increased gastric emptying in healthy humans and in patients after colo-rectal surgery. There was no effect on small bowel or colonic transit in healthy humans, but time to defecation was shorter when ghrelin was administered after colo-rectal surgery. Post-prandial elevations of glucose, insulin and GLP-1 occurred earlier and were higher with ghrelin in healthy humans. Plasma concentrations of ghrelin were unchanged after RYGB. Gastric (pouch) emptying was twice as fast after RYGB compared to before surgery. There was a progressive increase in several GI peptides after RYGB over 1 year after surgery.

Conclusions

In rodents ghrelin stimulates GI motility but obestatin has no effect. Ghrelin potently stimulates gastric emptying in healthy humans and in patients after surgery, while stimulation of intestinal motility seems more outspoken after surgery. In contrast to other studies plasma ghrelin was only significantly different on day 3 and thereafter not different after RYGB. Even if exogenous ghrelin can stimulate motility, endogenous ghrelin does not change after surgery and is less likely to modulate GI motility, transit or glucose metabolism after RYGB. However, early changes of other GI peptides (e.g. glucagon-like peptide-1) after RYGB likely contribute to the improved glucose homeostasis post surgery.

LIST OF PUBLICATIONS

I. Bassil AK, Häglund Y, Brown J, Rudholm T, Hellström PM, Näslund E, Lee K, Sanger GJ.
Little or no ability of obestatin to interact with ghrelin or modify motility in the rat gastrointestinal tract
Br J Pharmacol 2007;150:58-64.

II. Falkén Y, Hellström PM, Sanger GJ, Dewit O, Dukes G, Grybäck P, Holst JJ, Näslund E.
Actions of prolonged ghrelin infusion on gastrointestinal transit and glucose homeostasis in humans
Neurogastroenterol Motil. 2010;22:192-200.

III. Falkén Y, Webb DL, Abraham-Nordling M, Kressner U, Hellström PM, Näslund E.
Intravenous ghrelin accelerates postoperative gastric emptying and time to first bowel movement in humans
Neurogastroenterol Motil. 2013: in press

IV. Falkén Y, Hellström PM, Holst JJ, Näslund E.
Changes in glucose homeostasis after Roux-en-Y gastric bypass surgery for obesity at day three, two months, and one year after surgery: role of gut peptides
J Clin Endocrinol Metab. 2011;96:2227-35.

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LIST OF ABBREVIATIONS

AgRP	Agouti protein related peptide
AUC	Area under the curve
ARC	Arcuate nucleus
ACh	Acetylcholine
BBB	Blood-brain barrier
BMI	Body mass index
CCK	Cholecystokinin
CNS	Central nervous system
DPP-4	Dipeptidyl peptidase 4
EFS	Electrical field stimulation
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric nerve system
ERAS	Enhanced recovery after surgery
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic polypeptide
GH	Growth hormone
GHS-R	Growth hormone secretagogue receptor
GLP-1	Glucagon-like peptide-1
GOAT	Ghrelin o-acyl transferase
GRPP	Glicentin-related pancreatic polypeptide
GTP	Guanosine triphospate
HOMA-IR	Homeostasis model assessment of insulin resistance
HPLC	High-pressure liquid chromatography
IBS	Irritable bowel syndrome
IP-1	Intervening peptide-1
IV	Intravenous
MMC	Migrating motor complex
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
PEG	Polyethylene glycol
POI	Postoperative ileus
PP	Pancreatic polypeptide
PYY	Peptide YY
RYGB	Roux-en-Y gastric bypass surgery
VAS	Visual analogue scale

1 INTRODUCTION

1.1 ANATOMY AND PHYSIOLOGY OF THE GASTROINTESTINAL TRACT

1.1.1 **Stomach**

The stomach is anatomically divided into separate parts (cardia, fundus, corpus, antrum) and ends with the pyloric sphincter muscle before the duodenum. Circular and longitudinal muscles cooperate to grind and move the food into the duodenum. The rate of delivery of nutrients from the stomach to the duodenum is 1-4 kcal min^{-1} in healthy individuals. Depending on the caloric content and liquid or solid food content the gastric emptying rate will differ. The small intestine can influence the gastric emptying rate by a negative feedback mechanism. Peptide hormones secreted from the intestine (mainly the ileum), such as glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) inhibit gastrointestinal (GI) motility and are involved in the so-called "ileal brake" mechanism, where signals from the lower GI tract inhibit the upper GI tract.

The wall of the stomach contains four layers where the inner layer (mucosa) is protected by mucous produced from goblet cells. Other cells such as parietal cells secrete hydrochloric acid or intrinsic factor (for absorption of vitamin B12) and chief cells that secrete pepsinogen (precursor to pepsin that degrades proteins). Furthermore, there are enteroendocrine cells which secrete peptide hormones such as gastrin, ghrelin and obestatin. GI motility is controlled both by the local myenteric plexus (situated between the circular and longitudinal muscle layers), the vagal and sympathetic nerve system, as well as, by peptide hormones.

1.1.2 **Small bowel**

The small intestine is approximately 4 to 7 meters long, divided into the duodenum, jejunum and ileum and ends with the ileocecal valve, which prevents backflow from the colon. The layers of the small intestinal wall include the mucosa, submucosa, muscular layers and serosa. The main function of the small intestine is the absorption of nutrients, transport of chyme and indigestible materials to the colon. The small intestine is innervated by the vagus nerve and nerves from the spinal cord, as well as, the enteric nerve system (ENS). The enteric nervous system involves two separate collections of neurons located in the gut wall:

- The myenteric plexus situated between the circular and the longitudinal muscle layers which mainly regulates the motility of the GI tract.
- The submucosal plexus which regulates the blood flow of the intestinal wall and villus motility.

There are two recognisable motility patterns in the small bowel; peristalsis and the migrating motor complex (MMC) [1]. Peristalsis is stimulated by mechanical and chemical stimuli and is mainly dependent on the ENS. The MMC consists of distinct phases with different defined patterns of contractility, propagation and regularity. The MMC cycle length varies between different species, but in fasting humans it recurs approximately every 90-120 minutes (from stomach to distal ileum) [2] and consists of 3 phases (in rodents it occurs about every 15 min (Fig 1):

I. A period of absence of contractile activity lasting 45-60 minutes.
II. A period of 30 minutes charactericed by irregular contractile activity.
III. Rapid peristaltic contractions lasting 5-15 minutes.

Phase III is usually easy to recognise and is often measured in the proximal jejunum (Fig 1). Different phases of the MMC may occur in different parts of the small bowel and do not always reach the terminal ileum. If food is ingested, the motility pattern becomes irregular again. It has been proposed that this interdigestive peristalsis has a "housekeeping role" in cleaning the lumen of the bowel thereby preventing bacterial overgrowth.

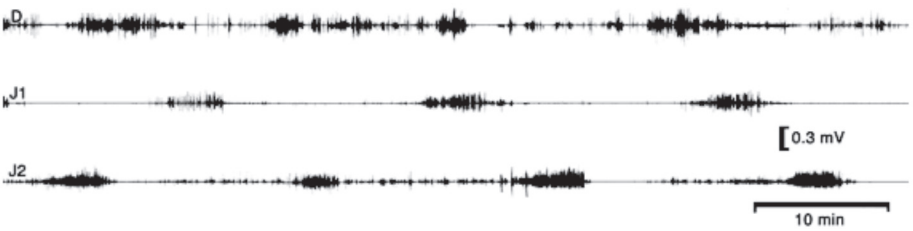


Fig 1. A representative recording of small bowel electrical activity in the rat (MMC) with defined phase IIIs (activity fronts).

1.1.3 **Colon and rectum**

The total length of the colon is about 1 meter and is divided into the cecum, ascending, transverse, descending and sigmoid colon. The rectum is usually defined as the last 15 cm proximal to the anal verge. The wall of the colon also includes an inner and outer smooth muscle layer with the myenteric nerve plexus situated in between. Movements of the colon are less frequent and the volume and consistency of the contents affect the rate of emptying [3].

1.2 GASTROINTESTINAL PEPTIDE HORMONES

1.2.1 **Background**

Over 30 different peptide hormones are secreted from the GI tract and numerous of these regulate GI motility, as well as energy homeostasis. Some peptides are produced peripherally and others also centrally and are therefore sometimes referred to as members of the "gut-brain axis" [4]. A number of studies have proposed associations between GI peptides and diseases such as irritable bowel syndrome (IBS) [5, 6], obesity [4, 7, 8], anorexia nervosa [9], postoperative ileus (POI) [10] and diabetes [11].

1.2.2 **Ghrelin and its receptor**

Ghrelin is a 28-amino peptide derived from a larger peptide containing 117 amino acids [12] (Fig 2). The peptide was identified as a ligand for the growth hormone (GH) secretagogue receptor (GHS-R) and therefore found to stimulate GH from the pituitary gland. Further studies show that the peptide is mainly produced in the stomach, but also in most human tissues examined (brain, small intestine, adrenal gland, gonads, pancreas, kidney) [13, 14, 15].

Ghrelin plasma levels are reduced up to 80% by gastrectomy [16]. The GHS-Rs are expressed throughout the GI tract [17], as well as, in the heart, lung, kidney, pancreas and adipose tissue. In the brain, GHS-Rs are found both in areas within and outside the blood-brain-barrier (BBB) including the hypothalamus, arcuate nucleus (ARC) [18], the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus. A recent study found that brain areas involved in reward and motivation (amygdala, frontal cortex) were signalling when ghrelin was given intravenously to healthy volunteers during functional magnetic resonance imaging [19].

Fig 2. Ghrelin and obestatin are derived from the 117-amino-acid peptide prepro-ghrelin.

1.2.3 **Structural variants of ghrelin**

Ghrelin circulates in acylated and des-acylated forms [12]. Both variants have recently found to be biologically active, but with different and even counteracting effects [20]. The ghrelin peptide is acylated by the enzyme GOAT (ghrelin O-acyl transferase) which is highly expressed in the stomach, pancreas and GI tract. The acylation is necessary for binding to the ghrelin receptor. Des-acylated ghrelin (which is found to be more than 2.5 higher in serum) exert its activity on a still unknown receptor. Studies in animals have shown that acylated ghrelin stimulates secretion of growth hormone (GH) from the pituitary [21], gastric motility and appetite resulting in increased bodyweight and consequently adiposity and increased insulin resistance. On the other hand des-acylated ghrelin counteracts the effect of acylated ghrelin and acts as a satiety signal by decreasing food intake and gastric emptying [22] [23]. This peptide also differs from acylated ghrelin by increasing the uptake of glucose and fatty acids in peripheral tissues, thus improving insulin sensitivity [24].

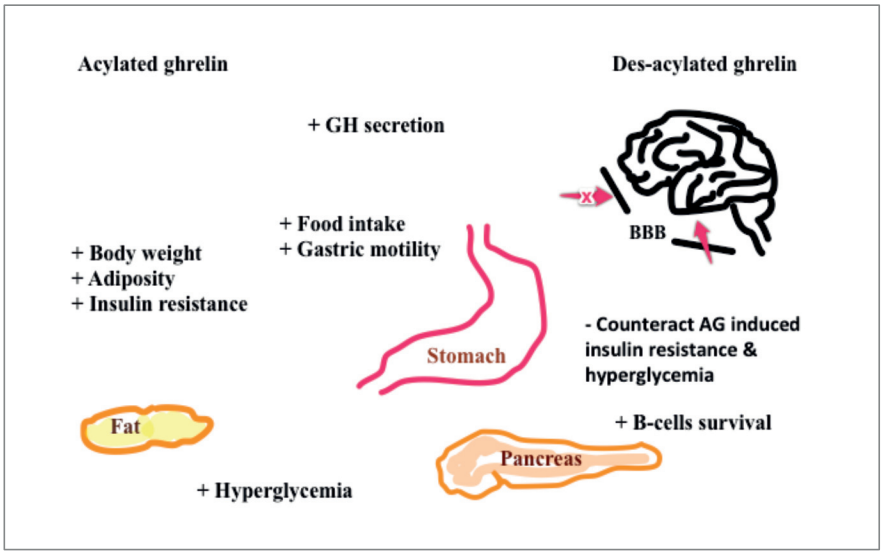


Fig 3. Some effects of acylated (AG) and des-acylated ghrelin in different organs.

In vivo studies of rat gastro-duodenal motility have further shown that des-acyl ghrelin exerts inhibitory effects on gastric motility, but not on duodenal motility in the fasted state [25]. The communication between the periphery and brain seems to differ between the peptides as des-acyl ghrelin does not activate the vagal pathway, but acts directly on receptors in the brain after crossing the BBB in contrast to acyl-ghrelin (Fig 3) [25].

1.2.4 **Ghrelin and gastrointestinal motility**

GI motility is regulated by a complex network of peptide hormones and the nervous system. Historically, the most frequently studied GI hormones are CCK, secretin, gastrin and motilin. Other peptide hormones such as GLP-1, ghrelin, orexin, PYY and obestatin are receiving increasing attention. The ghrelin receptor was found to be structurally related to the motilin receptor [26] [27]. Both motilin and ghrelin stimulate gastric acid secretion [28] and emptying [29, 30]. In rat, the stimulatory effect of ghrelin on gastric emptying rate and small bowel motility has been extensively studied. Both resting muscle tension and nerve-mediated responses evoked by electric field stimulation (EFS) are increased by ghrelin [17, 31, 32]. *In vivo*, ghrelin induces MMC activity both in the rat [33, 34] and human. Some studies in rodents and humans have concluded that gastric motility is abolished by pharmacologically blocking the vagus nerve (atropine, capsaicin) or by surgical vagotomy. Others show contrasting results where ghrelin accelerates gastric emptying of a meal in vagotomised patients [35] and in diabetic gastroparesis [36].

Ghrelin acts both peripherally and centrally. Circulating peripheral ghrelin is released from the GI tract and studies in rodents have shown that the peptide reaches the brain by three mechanisms.

1. Directly via the bloodstream entering the brain where the BBB is lacking.
2. Directly by crossing the BBB [37].
3. Indirectly, via the vagus nerve.

Findings in rat experiments suggest that small amounts of ghrelin are also produced locally in the brain. In conclusion, peripherally as well as centrally produced ghrelin stimulates food intake in the regulatory parts of the brain where neurons that release neuropeptide Y (NPY) and AgRP (agouti protein related peptide) are activated.

1.2.5 **Ghrelin and energy homeostasis**

Ghrelin is secreted in a circadian pattern and plasma levels rise before meal-time [38] and fall postprandially usually within an hour. Interestingly, the levels rise as food is expected [38] whether or not the food is actually received [39] and a recently published study demonstrates that ghrelin levels even rise on visual presentation of a meal [40]. In rodents ghrelin acts as a meal initiator depending on the dark/light cycle [41]. It is not clear why ghrelin levels fall after a meal but the degree of reduction depends on the amount of ingested carbohydrates, proteins or fat [42] and it is necessary for the nutrients to reach the small bowel. Several studies demonstrate that both peripheral and central administration of ghrelin stimulates food-intake in rodents [43] and humans [44]. Vagotomy inhibits the increase in food intake following high doses of exogenous ghrelin [45, 46, 47]. Total ghrelin is low in obese individuals [48] and consequently elevated in humans with low body mass index (BMI) [49, 50, 51]. In summary, ghrelin regulates the body weight by modulating hunger and appetite, increasing GI motility and by signalling to different food regulatory areas in the brain.

1.2.6 **Role of ghrelin in glucose homeostasis**

It is well known that exogenously administered ghrelin increases glucose concentrations in plasma [24]. An increasing number of studies demonstrate that ghrelin suppresses insulin secretion [52]. Still the role of ghrelin in glucose homeostasis is not clear. Glucose homeostasis is a fine-tuned mechanism depending on both insulin secretion and insulin sensitivity, which reflects glucose uptake in liver, intestines, muscle and fat. There is also a complex regulation of glycogenolysis and gluconeogenesis in the liver and intestine. In addition, insulin secretion from islet β-cells in the pancreas is locally controlled by several different hormone producing cells; glucagon secreting α-cells, somatostatin producing δ-cells, pancreatic polypeptide (PP) secreting F-cells and ghrelin secreting P/D1-cells in humans and A-cells in rodents. More recently published data suggest that the major metabolic role of ghrelin is to maintain stable blood glucose levels in spite of low energy intake [38, 50, 53, 54]. Animal studies have shown that ghrelin knock-out mice have improved glycemic control by enhanced insulin secretion [53], but are not able to maintain the same blood glucose levels compared to wild-type littermates during low calorie intake [55].

1.2.7 **Obestatin**

Obestatin is derived from the same precursor gene as ghrelin (Fig 2). It was initially thought to bind to the orphan G protein coupled receptor GPR39. Early reports [56] proposed that the peptide had opposite effects of ghrelin and therefore inhibited food ingestion and GI motility. It was also described that the rate of gastric emptying in GPR39 knockout mice was faster [57]. Later studies including ours [58, 59, 60, 61] have questioned these findings and suggest that obestatin binds to a still undiscovered receptor and may have other functions than affecting GI motility. Studies of rodents have proposed that obestatin has several biological roles from promoting sleep and memory to involvement in fluid homeostasis [62, 63, 64, 65].

1.2.8 **Peptide YY and pancreatic polypeptide**

PYY and PP belong to the NPY family of peptides. PYY itself has two different forms, PYY 1-36 and PYY 3-36. PYY 3-36 is the major form and cleaved from PYY 1-36 by the enzyme dipeptidyl peptidase-4 (DPP-4) in the circulation. PYY levels are low in the fasting state but rise after a meal and remain elevated for 1-2 hours depending on the amount of calories ingested [66]. Intake of fat stimulates PYY release more than proteins or carbohydrates. Both forms are secreted from the L-cells in the distal part of the GI tract with highest concentrations in colon and rectum (Fig 5). Low concentrations of PYY are also found in the brain. PYY and GLP-1 act as an "ileal brake" by inhibiting gastric emptying and secretion, gallbladder contraction and food intake [67, 68]. PYY also affects glucose homeostasis by increasing insulin-stimulated glucose uptake in peripheral tissues, and low levels of PYY are associated with elevated insulin resistance in humans [69].

PP is secreted from the pancreas (Fig 5), is released after food intake and inhibits gastric emptying [70]. It is also known to regulate glucose homeostasis by reducing the release of glucose as well as increasing the number of insulin receptors in the liver [71]. Both PP and PYY activate Y receptors (Y_1- Y_5) expressed in the liver, adrenal gland, intestines and brain.

1.2.10 **Somatostatin**

Somatostatin is a hormone located in most parts of the brain as well as in peripheral endocrine organs including pancreas, intestines, thyroid, kidneys and placenta. The hormone exists in two forms with 14 or 28 amino acids and is produced in the δ-cells of the intestine and the pancreas. The hormone acts as an inhibitor in most biological systems of the body and regulates GI motility and secretion, absorption of nutrients and smooth muscle tone in blood vessels as well as GI peptide hormone release. The release of several GI hormones including gastrin, cholecystokinin (CCK), insulin and glucagon are inhibited by somatostatin. Studies in healthy and obese subjects indicate that also ghrelin release is suppressed by somatostatin [72, 73].

1.2.11 **GLP-1, GLP-2 and glucagon**

The precursor peptide proglucagon is cleaved to organ specific peptides. Glicentin, consisting of 69 amino acids [74], is processed into glucagon and glicentin-related pancreatic polypeptide (GRPP) in the pancreatic α-cells. Another large molecule from the proglucagon is formed in the pancreas and named the major proglucagon fragment. A different process takes part in the brain or in the L-cells of the intestine where glicentin may be cleaved to GRPP and oxyntomodulin. The proglucagon fragment consists instead of two glucagon-like peptides (GLP-1 and GLP-2) and an intervening peptide (Fig 4). GLP-1 is even further processed in the L-cells resulting in GLP-1(7-37) and GLP-1(7-36)amide. GLP-1(7-37) usually exists in rats. The L-cells are found both in the small intestine and colon but are mainly present in the ileum (Fig 5) [75]. The cells have microvilli protruding into the intestinal lumen and can thereby "analyse" the food content and regulate the peptide secretion. The mucosa of the intestine; however, also contain the enzyme DPP-4 which degrades GLP-1 almost immediately. The peptide is further degraded in the liver and by enzymes lining the walls of blood vessels resulting in a half-life of GLP-1 in plasma of 1-2 minutes [76]. Apart from the intestines, GLP-1 is synthesized in neurons in the brainstem (NTS) and hypothalamus [77]. GLP-1 receptors are distributed in the kidney, pancreas, brain, heart and the GI tract [78].

GLP-1 release is stimulated by luminal carbohydrates and lipids [79]. Increased blood sugar also stimulates the release of the hormone, which in turn slows gastric emptying and intestinal motility. The secretion is inhibited by somatostatin, independent of the vagal nerve. In contrast to these findings, the effect of GLP-1 on gastric motility seems to be driven by the vagus nerve [80]. The pancreas is another important target organ for GLP-1 as it inhibits glucagon secretion and stimulates the release of insulin. Interestingly, studies of rodents and human pancreatic tissue have shown that GLP-1 stimulates growth and survival of β-cells [81, 82]. GLP-1 analogues and DPP-4 inhibitors have been introduced as a treatment of diabetes type 2. The side effects are few and treatment with GLP-1 does not lead to hypoglycaemia. Other positive effects beside increased insulin secretion are reduced appetite and a moderate weight loss. GLP-2 is also produced in the L-cells of the GI tract but does not seem to regulate motility or insulin secretion. It acts more as a growth factor stimulating intestinal growth such as increased cell proliferation and blood flow [83]. Studies in rodents have shown that GLP-2 inhibits apoptosis of enterocytes and in colectomised patients villus height is increased resulting in better absorption capacity of the small bowel [84].

Glucagon is secreted from the pancreas (Fig 5) and glucagon receptors are expressed mainly in the liver and kidney, but also in the pancreas, brain, heart, eye and GI tract. Glucagon is extensively studied as a regulatory hormone of the glucose homeostasis preventing hypoglycaemia and counteracting the effects of insulin. To prevent low glucose levels, glucagon stimulates gluconeogenesis and glycogenolysis in the liver. The hormone has additional metabolic functions as it has been shown to lower food intake by decreasing meal size in humans [85] and further involvement in the lipid metabolism.

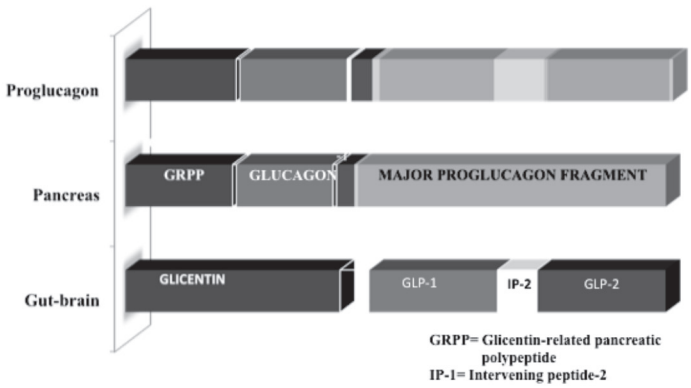


Fig 4. Schematic presentation of the precursor peptide proglucagon processed to different peptides depending on organ.

1.2.12 **Glucose-dependent insulinotropic polypeptide**

Glucose-dependent insulinotropic polypeptide (GIP) and GLP-1 are recognised as incretins. Both are regarded as equally important but GIP is circulating at 10-fold higher concentrations in plasma. Both glucose and fat intake [79] stimulate GIP release from the K-cells in the duodenum and jejunum (Fig 5). GIP increases insulin secretion in humans in response to elevated glucose levels. A feedback mechanism exists as circulating insulin also affects the synthesis and secretion of GIP [86].

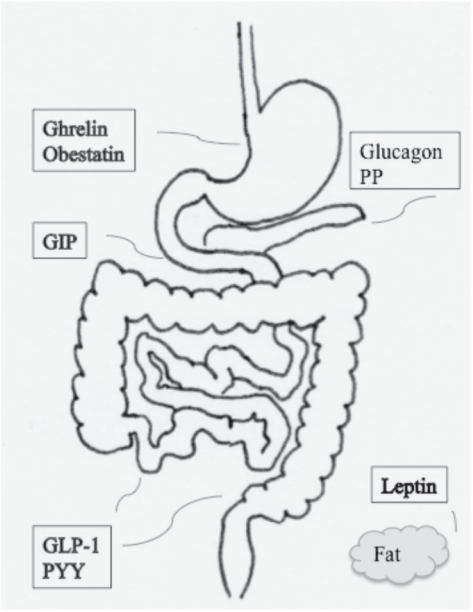


Fig 5. The main sites of GI peptide hormone secretion

1.3 **LEPTIN**

Leptin is an adipokine mainly found in adipose tissue but also in the stomach, breast and gonads. Plasma leptin concentrations strongly correlate with BMI and are therefore increased in obese patients [87]. Although leptin levels are low during the fasting state and increase gradually after a meal [88] it is not clear if it is regulated by meals or by the amount of fat storage. When weight gain occurs, leptin levels rise and consequently suppresses production and secretion of neuropeptide Y [89] within the ARC. This in turn leads to reduced appetite and food intake.

2 AIMS

The aim of this thesis was to investigate the role of ghrelin and obestatin in GI motility *in vitro* and *in vivo* in rodent and man. More specifically the aims were to investigate:

- The role of obestatin in GI motility in vitro and in vivo in rat and the interaction of ghrelin and obestatin on GI motility.
- The effect of ghrelin on oro-caecal and colon transit in healthy humans.
- The effect of ghrelin on gastric emptying and post-operative ileus after colo-rectal surgery
- The effect of Roux-en-Y gastric bypass (RYGB) surgery for obesity on plasma ghrelin concentrations, GI peptides and gastric (pouch) emptying.

3 MATERIAL AND STUDY SUBJECTS

3.1 *IN VITRO* STUDIES OF RAT AND HUMAN TISSUE AND *IN VIVO* STUDIES OF RAT

Adult male Sprague-Dawley rats (250-250 g) were used in study 1. They were kept under standardized conditions on a commercial diet. During study periods they were housed singly and recovered at least 1 week after surgery was performed. All efforts were made to minimize the number of animals used and culling was done by overdose of pentobarbitone or by CO_2 asphyxiation followed by cervical dislocation.

3.2 SUBJECTS AND PATIENTS

3.2.1 **Study II**

Twelve men (age 26 ±1.2 years) participated in the study. The subjects were healthy and of normal weight (BMI 24.7 ±2.9 kg m^{-2}). They had no medical history of GI disease or abdominal surgery (except for appendectomy more than 1 year ago) and stated bowel habits within normal range (not less than 3 bowel movements per week or more than 2 per day). No medications including antibiotics or laxatives were used during the whole study period.

3.2.2 **Study III**

Twenty-four patients participated in the study (12 men and 12 women). Patients planned for bowel resection (cancer or diverticular disease) were recruited from the outpatient clinic. Exclusion criteria before start of study were patients with metastatic disease, age >80 years, pre-menopausal women or previous history of abdominal surgery except for appendectomy. They had no history of diabetes, pulmonary, kidney, liver or ischemic heart disease.

3.2.3 **Study IV**

Twelve patients (3 men and 9 women) planned for gastric by-pass surgery for obesity participated in the study. Recruitment was made from the elective waiting list for gastric by-pass surgery. Their mean age was 36 ±2 years and BMI was 45.3 ±1.9 kg m^{-2}. They were on no medication and had no history of diabetes.

4 METHODS

4.1 STUDY 1

4.1.1 **Ghelin GTPγS binding assay**

GTP gammaS is a G-protein activating analogue of guanosine triphosphate (GTP). Its labelled radio-ligand is used in G-protein receptor binding studies.

4.1.2 **Rat isolated tissues**

Sections of jejunum or stomach from adult male Sprague-Dawley rats were used in an organ bath for *in vitro* studies. Obestatin (100 nM) or vehicles were added for measurement of jejunal basal muscle contractility. To measure nerve-mediated contractions of forestomach preparations, obestatin (0.01 nM-1 μM) or ghrelin (0.1 μM) were stimulated with EFS. To combine the two peptides and study the interactions between obestatin and ghrelin on EFS-evoked contractions, obestatin (0.01 nM-1μM) was added 1 min before 15 min application of ghrelin. Additionally, in separate experiments, 3 μM of prucalopride or ghrelin (0.1 μM) were added after applying obestatin. Results were expressed as percentage of mean amplitude of 10 μM acetylcholine-induced contractions.

4.1.3 **Rat GI motility *in vivo***

Three bipolar steel electrodes were implanted into the muscular wall of the small intestine (5, 15, 25 cm from pylorus) of male Sprague-Dawley rats (n=7) and for gastric emptying studies an additional indwelling polyethylene catheter was placed in the gastric forestomach. During experiments rodents were placed in Bollman cages (Fig 6) and electrodes were connected to an EEG preamplifier operating a Grass Polygraph. The MMC cycle length, propagation velocity and duration were measured during fasting and fed conditions. Three sets of experiments of the effect of ghrelin or saline on MMC were performed:

I A bolus of 2 ml of Isosource (1 kcal ml^{-1}) was administered through the gastric catheter during 3 min. Ten minutes later, either saline or ghrelin (300, 1000 or 3000 pmol kg^{-1} min^{-1}) was administered intravenously (IV) at a rate of 5-45 μl min^{-1} until fasting motility pattern of MMC returned.

II After a 60 min period of control, ghrelin (1000 pmol $kg^{-1}min^{-1}$) or saline were given (15 μl min^{-1}) continuously over 4 hours. MMC cycles with fasting phase III motility pattern was measured.

III For a period of 6 hours, saline or ghrelin (1000 pmol $kg^{-1}min^{-1}$) were alternatively administered for 60 min and fasting motility pattern was studied.

To study the effects on fasting motility of obestatin alone or in combination with ghrelin, additional experiments were performed. Obestatin (1000 pmol $kg^{-1}min^{-1}$) or ghrelin (500 pmol $kg^{-1}min^{-1}$) were infused for 60 min. After a recovery period of two days the same rats that previously received the ghrelin infusion were now administered an infusion of obestatin (2500 pmol $kg^{-1}min^{-1}$) and 10 min later an additional infusion of ghrelin (500 pmol kg^{-1} min^{-1}). Both infusions continued for another 60 min.

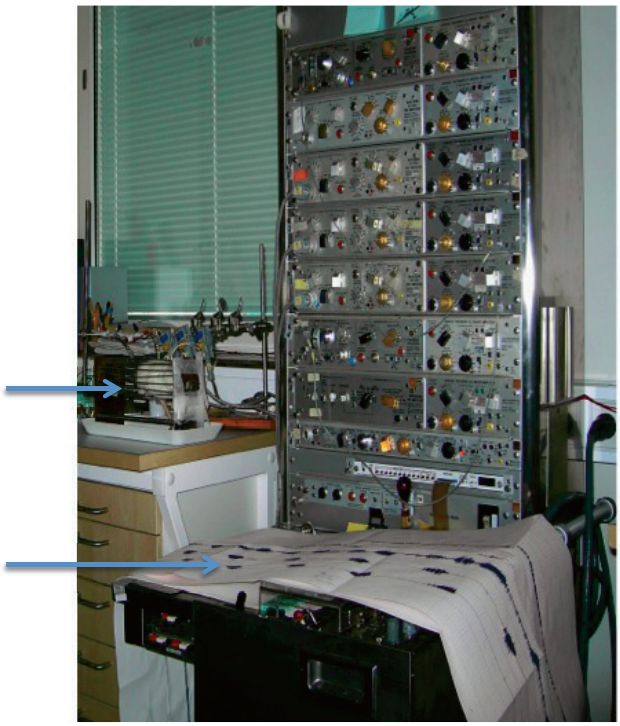


Fig 6. Rat in a Bollman cage (upper arrow) with electrodes connected to a Grass Polygraph 7B and a paper recording of the MMC (lower arrow).

4.1.4 Gastric emptying of rat

After an 18 hours fasting period, with free access to water, an infusion of obestatin (1000 and 30 000 pmol $kg^{-1}min^{-1}$) or saline was infused for 10 min. After the loading period, 0.5 ml of a radioactive solution (polyethylene glycol) or 50% of the polyethylene glycol (PEG) combined with an enteral feeding solution (Isosource) was administered for 30 s via the gastric catheter. The infusion of obestatin or saline continued for another 20 min. Thereafter the rats were killed, abdomen opened and the stomach removed after ligation of distal end of esophagus and the pylorus. The amount of remaining radioactive substance was calculated in a gamma counter for 60 seconds. Gastric emptying rate was calculated from the percentage of radioactive marker remaining in the stomach after 20 min compared to a standard of an equal volume of the radioactive marker.

4.2 STUDY II

4.2.1 **Human tissue**

Full thickness tissues were excised from surgical specimens in the operation theatre and immediately placed in modified Krebs solution. Six samples were taken from colon, gastric antrum and duodenum from patients undergoing surgery for cancer. The tissues were placed in organ bath and both basal and EFS muscle contraction was performed. Ghrelin in increasing concentrations was applied and results expressed as percentage of mean values of acetylcholine.

4.2.2 **Study design**

The design was a randomised double-blinded cross-over study with at least 2 weeks between the two-study periods. The study was conducted over three days and started the evening before the infusion day as the subjects swallowed capsules containing 20 radio-opaque markers. The following morning another capsule with 20 differently shaped markers was swallowed. Two hours later a calorie-fixed breakfast was served and an infusion of either saline or ghrelin (15 pmol $kg^{-1}min^{-1}$) was started. Together with breakfast, 1.5 g of acetaminophen and 15 ml of lactulose were ingested. During the day, at repeated intervals, plasma and breath samples were taken. Hunger, fullness and nausea were documented on a visual analogue scales (VAS) and a questionnaire regarding the bowel movements were filled out throughout the study period. The following day and 2 days after a plain X-ray of the abdomen was taken to calculate the number of remaining markers as well as the geometric index.

4.2.3 **Gastric emptying of humans**

The gastric emptying was assessed by the acetaminophen method [90]. The drug concentrations were converted to cumulative values and an absorption curve was made. Each individual absorption curve was inverted and transformed to a third-degree polynomial reflecting the gastric emptying profile. The T50 value (50% of the total cumulated dose emptied) was also calculated.

4.2.4 **Oro-caecal transit**

The breath hydrogen test was used to measure the transit time from oral ingestion of lactulose to arrival in the proximal part of colon. Briefly, lactulose (including lactose) is fermented by bacteria in the colon, where carbondioxid (CO_2) and hydrogen (H_2) is produced. The gases are absorbed by the colonic wall, transported via the veins to the lungs and exhaled via the breath. In our study alveolar breath samples were collected in bags every 10 min for 3 hours and immediately analysed by a Quintron Microlyzer. The oro-caecal time was defined as either an increase of hydrogen by at least 10 ppm or as doubling of hydrogen or methane concentrations. An early rise, or a high baseline level, may be a sign of small bowel bacterial overgrowth and was ruled out prior to the test. Patient instructions included fasting for 10 hours, no smoking or alcohol, avoidance of high-fiber containing meals and milk products.

4.3 STUDY III

4.3.1 **Study design**

The design was a randomised, double-blinded study performed at a single-centre with at least 4 days apart. The first study day took part in an outpatient clinic and second in the ward 2 days after surgery. Randomisation was done by envelope draw. On both occasions the study began in the morning at 9 am after an overnight fast and the subjects received either a 3-hour infusion of 0.9% saline or ghrelin (15 pmol $kg^{-1}min^{-1}$) IV. Before the infusion started, an oral nutritional drink with 480 kcal was ingested followed by 1.5 gram of acetaminophen dissolved in water. During the infusion blood samples were drawn repeatedly and VAS were completed for hunger, fullness and nausea. A patient questionnaire for bowel movements was filled in from first study day until discharge.

4.4 STUDY IV

4.4.1 **Study design**

The study was performed the day before and 3 days, 2 months and 1 year after RYGB surgery. After an overnight fast, the subjects swallowed 1.5 g acetaminophen together with a 200 ml liquid meal (300 kcal). Blood samples were taken before and at repeated intervals for 180 minutes. A standard RYGB with 1 meter Roux was performed laparoscopically in each patient.

5.0 ANALYSIS OF GLUCOSE, INSULIN AND GASTRO-INTESTINAL PEPTIDE HORMONES

Blood samples were collected in prechilled EDTA vacutainer tubes at different time-points. In study III a protease inhibitor cocktail together with DPP-4 inhibitor were added. Samples were centrifuged at 4^{o} C for 10 min at 3000 r.p.m.

5.1 **RIA (radioimmunoassay)**

In study II ghrelin (total) and GLP-1 were measured with a RIA kit. In study IV leptin, insulin, glucagon, GIP, somatostatin and GLP-1 was measured with a RIA kit.

5.2 **Multiplex immunoassay panel (Luminex system)**

In study II GIP, insulin, PYY and leptin were analysed with a multiplex immunoassay panel on a Luminex reader.

5.3 **Mutarotase and glucose dehydrogenase.**

In study II and IV glucose was measured by an enzyme assay (mutarotase and glucose dehydrogenase) with an YSI 2300 STAT Plus.

5.4 **Fluorescence-immunoassay**

In study II acetaminophen was assessed by fluorescence-immunoassay.

5.5 **Biochemistry analyser**

In study III plasma glucose levels and acetaminophen were measured by an automatic chemistry analyser (Architect C8000)

5.6 **HPLC (high-performance liquid chromatography)**

In study IV acetaminophen was measured by HPLC.

5.7 **ELISA (enzyme-linked immunosorbent assay)**

In study IV ghrelin (total) was measured with an ELISA kit.

5.8 **Electrochemiluminescence detection**

In study III human acylated ghrelin was analysed utilizing electrochemiluminescence detection (Meso Scale Discovery).

5.9 **Metabolic hormone panel bead-based immunoassay**

In study III GLP-1, GIP, insulin, PP and PYY were analysed using a human metabolic hormone panel magnetic bead-based immunoassay (MAGPIX, Millipore)

6.0 Assessment of insulin resistance with HOMA-IR

To measure the change of insulin sensitivity in study IV the homeostatic model assessment of insulin resistance (HOMA-IR) was used [91];

$$\frac{\text{Fasting insulin (μU/mL) x Fasting glucose (mmol/L)}}{22.5}$$

7.0 VISUAL ANALOGUE SCALE

In studies II-IV, during the infusion of ghrelin or saline, the subjects were at regular intervals asked to estimate their feeling of hunger, fullness and nausea. A visual analogue scale (VAS) of 100 mm was used (appendix). The intra (0-10 min) and inter-meal (+10 min until end of infusion or after breakfast to before lunch in study II) changes were calculated [92].

8.0 ETHICS

The regional Ethics committee of Stockholm approved the human studies I-IV including the collection of human tissue. Study II was also approved by the Radiation protection committee of the Karolinska University Hospital, Solna, Sweden. All subjects gave oral and written informed consent. Rat studies (study I) were approved by the animal care committee of UK and the Ethics Committee in northern Stockholm for the humane use of experimental animals in research.

9.0 DATA AND STATISTICAL ANALYSIS

For data acquisition and analysis of experiments involving rat tissues in vitro in study I, MP 100 hardware and AcqKnowledge were used. For statistical analysis and graph presentations GraphPad Prism (La Jolla, CA, USA), with continuously updated versions being used. Data are shown as mean ±SEM. The statistical significance of any differences between unpaired data was determined using Student's t-test or Mann-Whitney U test. For matched pairs Wilcoxon signed rank test was used (gastric emptying data, area under the curve) for peptides, oro-caecal and colonic transit and VAS. AUC was calculated using the trapezoidal rule. $P<0.05$ was considered as statistically significant.

10.0 RESULTS

10.1 STUDY I

10.1.1 **Ghrelin GTPγS binding assay**

Ghrelin showed a concentration-dependent increase in receptor binding ability. Obestatin alone or in combination with ghrelin showed no agonist or antagonist activity at the human ghrelin receptor.

10.1.2 **Rat isolated tissues**

Obestatin (100 nM) had no effect on baseline contraction on jejunal muscle strips or on nerve induced muscle contractions (EFS) in isolated forestomach tissue. In contrast to these findings, ghrelin (0.1 μM) increased the EFS-evoked contractions by 40% (42.7 ±7.8%) in rat forestomach. Combination of ghrelin and obestatin resulted in a significant inhibition of the excitatory response of ghrelin. However, the response was only achieved with obestatin in the two lowest concentrations (0.1 nM and 1 nM, $p<0.05$). In similar experiment with prucalopride instead of ghrelin as test substance, a larger increase of EFS-evoked contractions

was observed. When prucalopride was added to obestatin, no reduction of the excitatory response was found.

10.1.3 **Rat GI motility *in vivo***

During fasting the MMC cycle length was 15 ±0.8 min. When the nutritional solution with Isosource was administered, the return of fasting MMC pattern (phase III) at J2 was shortened by increasing concentrations of ghrelin (Table 1). The next sets of experiments of fasting rats infused with ghrelin (1000 pmol $kg^{-1}min^{-1}$) or saline during 4 hours shortened the MMC cycle length only the first hour compared to saline ($p<0.05$). The same results were found when ghrelin (1000 pmol $kg^{-1}min^{-1}$) or saline were given as alternating infusions for 6 hours.

Table 1. The effect of ghrelin on MMC (*=$p<0.05$).

Infusion (pmol $kg^{-1}min^{-1}$)	Time to phase III (min)
saline	39.4 ±1.9
ghrelin (300)	37.8 ±13.2
ghrelin (1000)	33.4 ±1.9*
ghrelin (3000)	29.5 ±0.5*

In the next set of experiments on fasting rats infused with ghrelin and obestatin, the control period of saline was 13.5 ±1.5 min. Obestatin had no effect on the MMC cycle length, duration of activity fronts or propagation velocity. As expected, ghrelin significantly shortened the MMC cycle length. Addition of ghrelin to obestatin did not significantly change the results from the saline infusion (Fig 7).

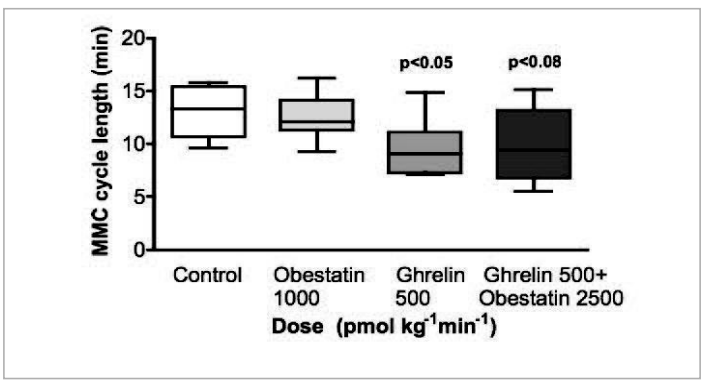


Fig 7. The effect of obestatin and ghrelin on MMC.

10.1.4 **Gastric emptying of rat**

During saline infusion the remaining radioactive non-nutrient PEG meal was 32.2 ±2.2 % and combined radioactive PEG and Isosource 35.4 ±2.3 % of the initially administered meal. There was no significant difference in gastric emptying of nutrient or non-nutrient liquid solutions when the infusion of obestatin (1000 or 30 000 pmol kg^{-1} min^{-1}) was given (34.1 ±2.2 % and 33.5 ±3.8; 31.3 ±2.1 and 34.8 ±3.0 %, nutrient and non-nutrient liquid, respectively for each dose studied).

10.2 STUDY II

10.2.1 **Human tissue in organ bath**

Experiments with muscle segments from human tissues did not show any contractile response when ghrelin was added in increasing concentrations (Fig 8). When EFS was used before and after application of ghrelin, gastric tissue (not duodenal or colonic) responded with an increase from 78 ±20 % to 125 ±30 % (of maximum response to ACh).

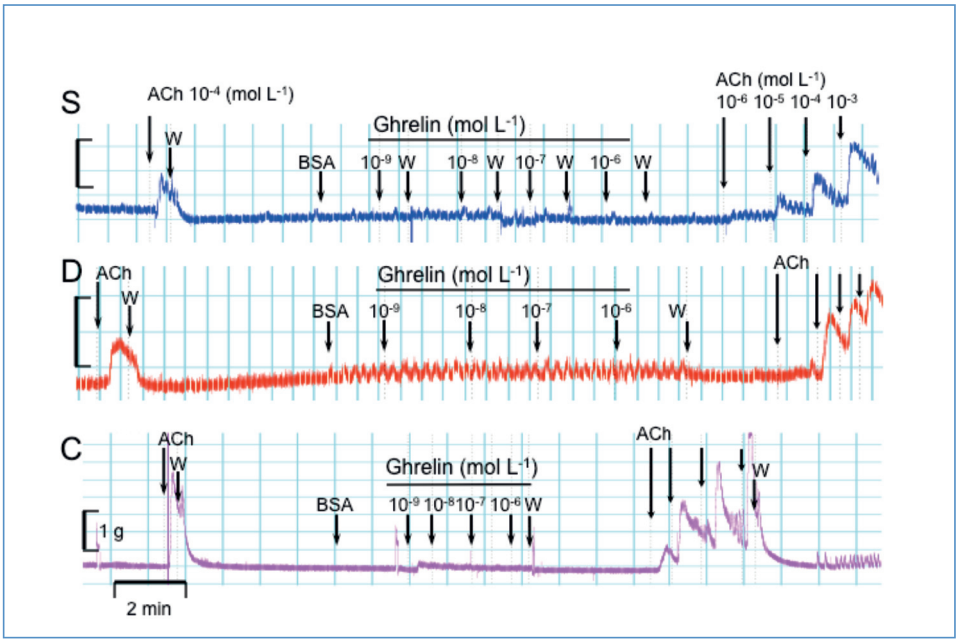


Fig 8. Increasing concentrations of ghrelin added to stomach (S), duodenum (D) and colon (C). Acetylcholine (ACh) was used to verify tissue viability and as positive control.
W = wash-out.

10.2.2 **Study subjects**

No adverse events were noticed during the study period and all subjects followed strictly the time schedule of study protocol with ingested markers and X-ray in similar way.

10.2.3 **Gastric emptying**

The gastric emptying rate was faster during ghrelin infusion compared to saline. The half emptying time was significantly shorter during ghrelin infusion (50.3 ±3.9) than during saline infusion (59.9 ±4.4 min) (p=0.002) (Fig 9).

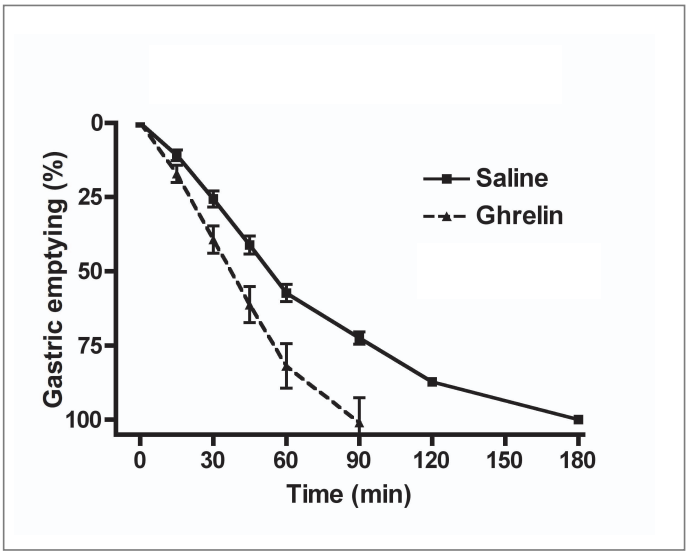


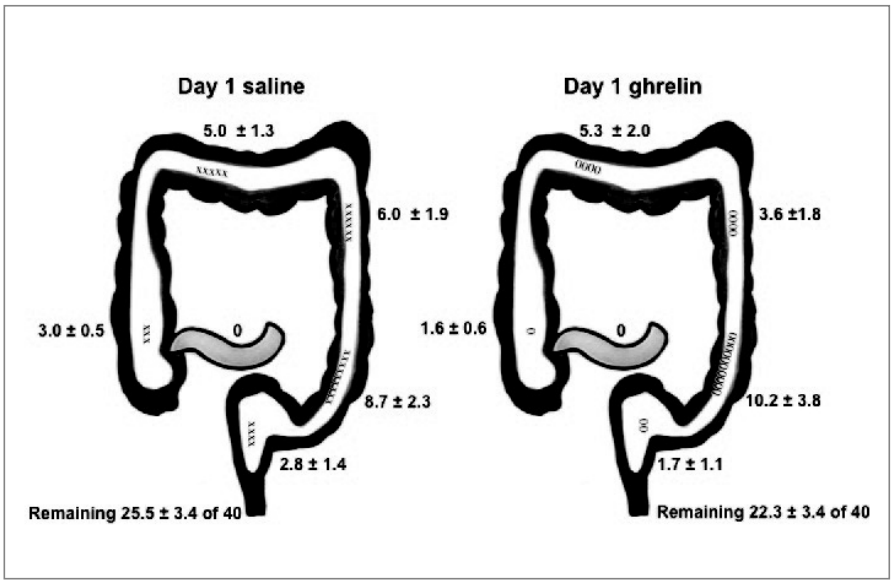
Fig 9. Gastric emptying rate in normal human volunteers using the acetaminophen method during an infusion of ghrelin (15 pmol $kg^{-1}min^{-1}$) or saline.

10.2.4 **Oro-caecal transit time**

The oro-caecal transit time was unchanged with ghrelin as compared to saline (40 ±7.8 min and 46 ±9.7 min, respectively).

10.2.5 **Colonic transit**

We found no differences of the remaining number of radio-opaque markers in the colon and no difference in distribution of defined segments of colon day 1 or day 2 (Fig 10).



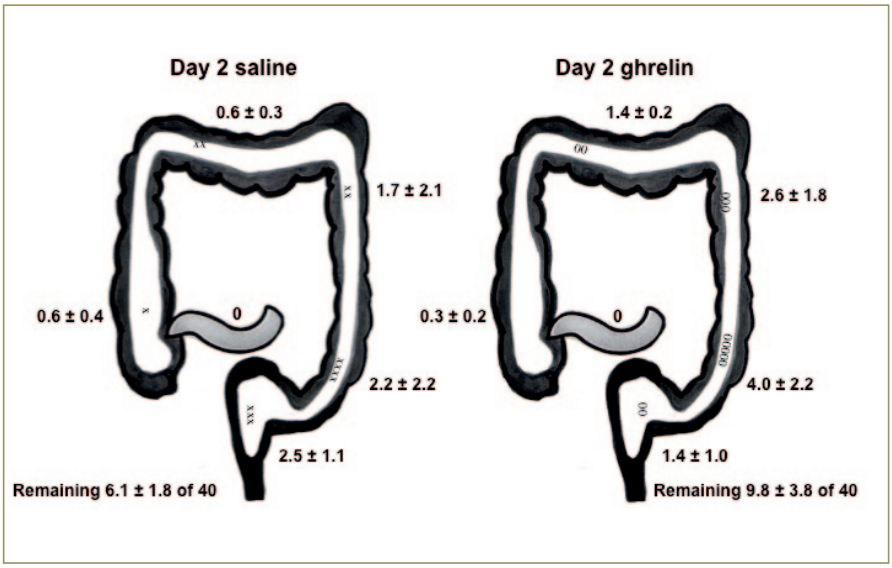


Fig 10. A schematic sketch of distribution (mean ± SEM) of radio-opaque markers.

Data from patient questionnaires revealed unchanged bowel movements by ghrelin infusion during all study days.

10.2.6 **Glucose homeostasis and GI peptide hormones**

The serum concentrations of ghrelin rose rapidly and reached a four-fold level with a maximum of 334 ±52 pmol L^{-1} at 120 min after the infusion of ghrelin was started. As expected, a decrease of endogenous ghrelin concentrations were observed after breakfast (Fig 11).

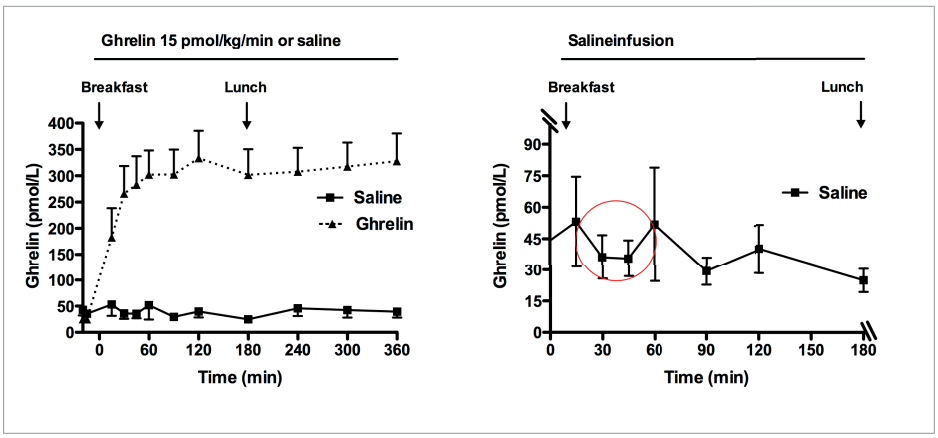


Fig 11. Plasma levels of ghrelin during infusion of ghrelin or saline. Postprandial decrease of ghrelin after breakfast meal encircled in red.

The infusion of ghrelin resulted in significantly elevated plasma glucose concentrations after breakfast and lunch meals compared to saline infusion ($AUC_{0\text{-}360}$ 2587 ±108 vs 1913 ±105 min mmol L^{-1}) ($p<0.001$). Consequently, plasma insulin was significantly higher during the

ghrelin infusion compared to saline (AUC_{0-360} 50678 ±4002 vs 33588 ±4273 min pmol/L) ($p< 0.001$).

The insulinogenic index was not significantly different (17.5 ±1.9 vs 19.7 ±1.5 saline and ghrelin, respectively). Plasma GLP-1 was significantly elevated during ghrelin infusion (AUC_{0-360} 8717 ±588 and 6609 ±552 min pmol L^{-1}, p=0.001). The plasma levels of GIP, PYY and leptin did not differ during saline or ghrelin infusions.

10.2.7 **Visual analogue scale**

Hunger ratings were higher throughout the ghrelin infusion as compared to saline. The inter-meal (after breakfast to before lunch) change in hunger rating was significantly higher during ghrelin infusion (40 ±5 vs 23 ±4) ($p<0.05$). Similarly, ratings of fullness were lower throughout the ghrelin infusion compared with saline, but the inter-meal change in fullness was not different (33 ±6 vs 26 ±5). Nausea ratings were low and not different between ghrelin and saline infusions.

10.3 STUDY III

10.3.1 **Patients**

Twenty-two patients were enrolled for the study. Four patients were excluded. Two were incidentally administered acetaminophen for pain relief the night prior to the study and were not studied postoperatively. One patient showed early signs of anastomotic leakage and one patient could not consume the liquid meal and were therefore not studied postoperatively. Eleven patients remained in the saline group (7 men) and 9 patients (3 men) in the ghrelin group (table 4, 5). Heart rate and blood pressure remained stable during ghrelin and saline infusions.

Table 4. The study group that received saline infusion.

Age	BMI	Surgery	Hospital stay	Tfb	OR time	Blood loss
66	24,8	sr	5	1	109	150
78	28,1	rh	5	2	176	400
67	24,7	rh	4	3	157	600
79	27,5	apr + stoma	8	5	328	1100
56	34,1	rh	3	4	105	150
62	24,6	sr	29	5	135	150
68	24,8	rr + stoma	6	5	233	550
65	36,1	sr	14	3	127	300
81	21,8	sr	9	4	222	450
74	21,9	sr	3	3	111	75
68	28	apr + stoma	7	3	292	300

rh=right hemicolectomy, rr=rectal resection, lr=left hemicolectomy, sr=sigmoid resection, apr=abdominoperineal resection

Table 5. The study group that received ghrelin infusion. Time to first bowel movements (Tfb). Operating room (OR).

Age	BMI	Surgery	Hospital stay	Tfb	OR time	Blood loss
73	21	rh	3	3	138	100
69	22,8	rr + stoma	8	2	163	200
75	24,1	lr	4	2	180	100
64	24,9	rr	4	1	180	100
73	26,7	lr	3	2	180	150
62	24,2	sr	4	2	155	100
63	23,6	sr + stoma	3	2	157	400
64	26,9	sr	6	1	172	300
66	30.9	apr + stoma	25	4	138	1550

10.3.2 **Gastric emptying**

Results from analysis of plasma acetaminophen were obtained from pre- and postoperatively comparisons of the same individuals but also between the groups. There was no difference of the AUC acetaminophen during the pre- and postoperative period during the ghrelin infusion (p=0.57). The study subjects that received the infusion of saline had a slower gastric emptying rate after surgery (p=0.02). Comparisons between study groups both before and after surgery indicated an increased gastric emptying rate as AUC acetaminophen was significantly higher with ghrelin infusion compared with saline both before and after surgery (p=0.02 and 0.01, respectively) (Fig 12).

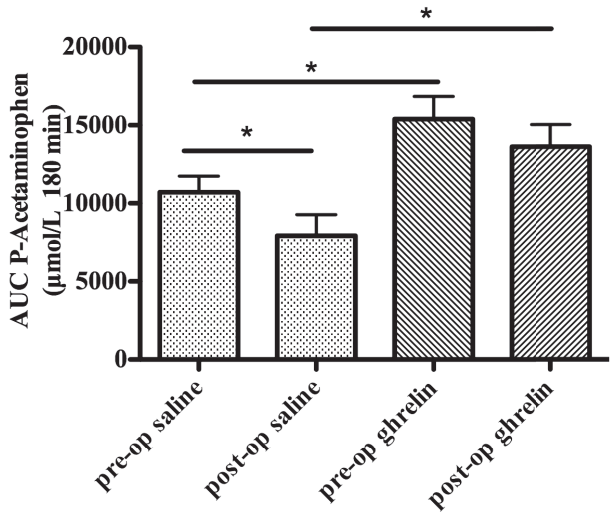


Fig 12. The $AUC_{0\text{-}180}$ for acetaminophen before and after surgery with saline (control) and before and after surgery with ghrelin (* = $p<0.05$).

10.3.3 **Active ghrelin**

Analysis of active (acylated) ghrelin confirms that supraphysiological doses of ghrelin were administered IV. A 40-fold elevation of ghrelin was measured during ghrelin infusion compared to saline. There was no difference in $AUC_{0\text{-}180}$ acyl ghrelin during the pre- and postoperative infusion of ghrelin.

10.3.4 **Plasma concentrations of glucose and insulin**

There was no difference in the $AUC_{0\text{-}180}$ glucose between the pre- and postoperative saline infusion or the ghrelin infusion. However, the infusion of ghrelin led to higher $AUC_{0\text{-}180}$ glucose both before and after surgery compared to the values obtained during the saline infusion ($p=0.0003$ and $p=0.003$). $AUC_{0\text{-}180}$ insulin was not different during saline infusion pre- and postoperatively ($p=0.10$). In contrast to these findings, $AUC_{0\text{-}180}$ insulin was significantly higher during the preoperative ghrelin infusion compared to the postoperative ghrelin infusion ($p=0.004$). There was no significant difference between $AUC_{0\text{-}180}$ insulin during ghrelin infusion compared to saline before and after surgery ($p=0.08$ and 0.17, respectively).

10.3.5 **Visual analogue scale**

The inter-meal change in hunger rating was 23.4 ±5.7 and 8.1 ±5.1 for saline infusion before and after surgery, respectively. The change during ghrelin infusion was 35.1 ±15.2 and 34.4 ±11.3 before and after surgery, respectively. The only significant difference was between saline and ghrelin infusion post-operatively ($p=0.05$). There was no difference in fullness or nausea ratings before and after surgery during saline and ghrelin infusion.

10.3.6 **Plasma concentrations of GLP-1, GIP, PP, PYY**

There were no differences in $AUC_{0\text{-}180}$ of GIP, GLP-1, PP and PYY during saline or ghrelin infusion before or after surgery. The $AUC_{0\text{-}180}$ of these peptides was not different between ghrelin and saline before and after surgery.

10.4 STUDY IV

10.4.1 **Subjects**

A significant weight loss was reached at 2 months ($p=0.003$) and 1 year ($p=0.003$).

10.4.2 **Analysis of acetaminophen absorption (gastric and pouch emptying)**

The absorption of acetaminophen reflecting gastric or pouch emptying was significantly different after RYGB compared to before. The time to maximum plasma concentrations of acetaminophen was almost immediate after intake of liquid meal compared to pre-operative levels (61.9 ±5.0 min). The half emptying time of acetaminophen was also halved after surgery and remained constant over time (Fig 13).

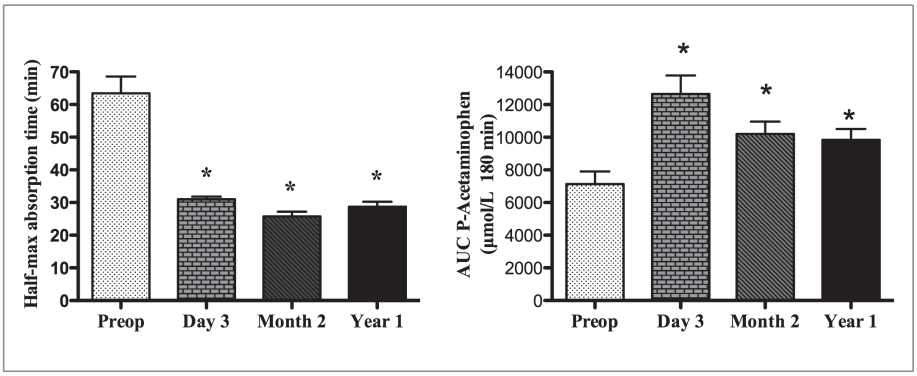


Fig 13. Half-maximum absorption time and AUC of acetaminophen (* = $p<0.05$).

10.4.3 **Analysis of fasting glucose and insulin**

Fasting plasma glucose concentrations remained lower compared to preoperative values during the whole study period. Consequently, plasma insulin was significantly decreased at 2 months and 1 year. HOMA-IR was also reduced at 2 months and normalized at 1 year (Fig 14).

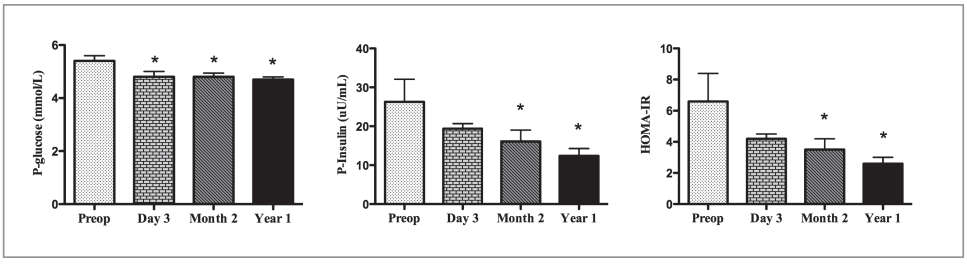


Fig 14. Fasting plasma concentrations of glucose and insulin (* = $p<0.05$)

10.4.4 **Analysis of fasting pancreatic glucagon, enteroglucagon, GLP-1 and GIP**

Plasma concentrations of total glucagon were measured in plasma. Fasting plasma levels of pancreatic glucagon were unchanged at all time points. Enteroglucagon levels (result of subtraction of pancreatic glucagon from total glucagon) were significantly decreased only on day 3. Fasting GLP-1 was also unchanged. GIP was significantly decreased at day 3 and 1 year after surgery. Plasma somatostatin was not significantly changed at different study points compared with before surgery.

10.4.5 **Analysis of fasting leptin and ghrelin**

Fasting levels of ghrelin were unchanged at all time-points. Leptin as expected was significantly reduced after 2 months and 1 year.

10.4.6 **Analysis of post-prandial glucose and insulin**

The whole curve was shifted to the left even though the peak of glucose occurred at 30 min both before and after surgery. The RYGB surgery resulted in an earlier rise and steeper decline of plasma glucose. Insulin followed a similar pattern with peak concentrations at 20 min and a leftward shift of the curve. $AUC_{0\text{-}180}$ for glucose and insulin were unchanged throughout the study period.

10.4.7 **Analysis of post-prandial pancreatic glucagon, enteroglucagon, GLP-1 and GIP**
Plasma levels of pancreatic glucagon were elevated at 2 months but were unchanged from pre-operative values at 1 year. Enteroglucagon, GLP-1 and GIP showed a similar pattern with leftward shift of plasma concentrations after surgery and a progressive rise over time. Somatostatin showed a rightward shift with a peak appearing later. Pancreatic glucagon $AUC_{0\text{-}180}$ was increased on day 3 and 2 months but not at 1 year after surgery. $AUC_{0\text{-}180}$ for enteroglucagon and GLP-1 were significantly increased both at day 3, 2 months and 1 year. $AUC_{0–180}$ for GIP were unchanged pre- and postoperatively. Somatostatin $AUC_{0–180}$ was significantly decreased on day 3 only and then returned to baseline AUC at other study points.

10.4.8 **Analysis of post-prandial leptin and ghrelin**
Plasma ghrelin $AUC_{0\text{-}180}$ was significantly lower on day 3 after surgery but not significantly different at 2 months and 1 year. Leptin AUC 0-180 min was significantly lower at 2 months and 1 year (Fig 15).

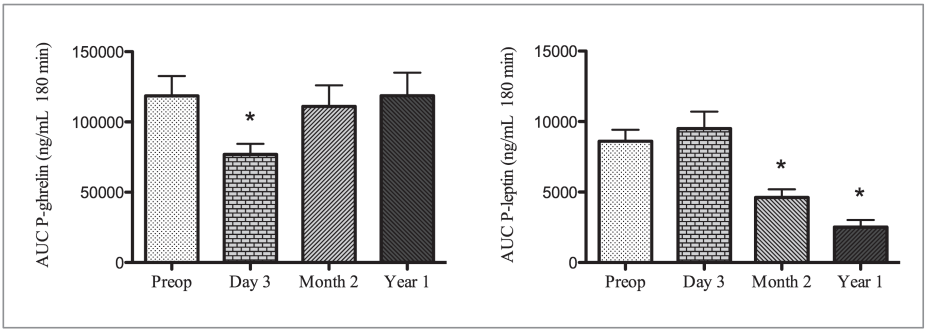


Fig 15. $AUC_{0\text{-}180}$ minutes of ghrelin and leptin (* = $p<0.05$).

10.5 **Analysis of visual analogue scale**
Intake of a 200 ml liquid meal decreased the feelings of hunger on day 3, 2 months and 1 year. Similarly, fullness increased.

11.0 DISCUSSION

Focusing on ghrelin as an important regulator of GI motility we sought to clarify its role by different approaches, based on endogenous secretion and exogenous administration of the peptide as likely to be encountered in the clinical setting of surgery. The studies encompass investigations of ghrelin in animals as well as human experimental models, together with broad-based assays of GI peptide hormones known to be of regulatory importance for GI motility and metabolic control.

The aim of our first study included in this thesis was to further study the role of ghrelin in GI motility *in vitro* and *in vivo* in rodents and to investigate the ability of obestatin and ghrelin (alone or in combination) to affect the rat GI motility *in vitro* and *in vivo*. Thus, in part we tried to repeat, but also to extend the studies of Zang et al [56]; however, with little success.

Later studies clearly show obestatin to have no role in GI motility and questionable importance for body weight control [93].

The ability of ghrelin to hasten gastric emptying and stimulate MMCs in the rat and human is interesting in view of its metabolic consequences. Our study on ghrelin's effect on small bowel motility in rat confirmed previous findings, but also showed that the effect on MMC is diminished after an hour. A 4-hour infusion, as well as, alternating ghrelin or saline for 6 hours in fasting rats showed the same results. One explanation may be the degradation from active (acylated) to des-acylated ghrelin by the DPP-4 enzyme. There might also be a desensitization of the GOAT enzyme to activate circulating des-acyl ghrelin. Studies with peripherally administered des-acyl ghrelin have been shown to inhibit the fasting motility of the stomach [24], probably by crossing the BBB and acting centrally in the brain [25].

Our study showed very little effect of obestatin on GI motility and no interaction with ghrelin. The first study of obestatin by Zang et al proposed that the peptide decreased the gastric emptying rate and prevented body weight gain [56]. Furthermore, the author claimed that it decreased the contractility of jejunal strips *in vitro* and counteracted the effect of ghrelin. Our results could not confirm any significant effect of obestatin (from two different suppliers) on gastric emptying in a nutrient or non-nutrient setting nor the MMC pattern. Our only positive data of obestatin was on experiments with rat isolated gastric tissue where obestatin in the lowest dose range inhibited the excitatory response of ghrelin. In a follow-up study published in 2008 [94], the same author explained that the original peptide preparation was contaminated and some experiments could not be reproduced. However, the purified obestatin still showed binding properties to the GPR39, a receptor that belongs to the same family as the ghrelin receptor.

One interesting hypothesis by Depoortere and al [61] is that obestatin may be inhibited by endogenously secreted ghrelin. The effect of obestatin on gastric emptying and food intake was studied in fasted ghrelin knockout mice and wild types. They found no effect in either group of mice and concluded that even though endogenous ghrelin is abolished, no effect of obestatin is found on GI motility.

In summary, we could only show a modest effect of obestatin on gastric muscle contractility *in vitro* and no interaction with ghrelin. The results from our ghrelin studies are in line with those of others but also show a reduced effect after an hour.

Even though obestatin is derived from the same precursor gene as ghrelin its role might be of a different nature than that of ghrelin [93]: inducing cell proliferation of ovarian cells [95] and promoting survival of β-cells of the pancreas [96], inhibition of water drinking [63] and improving memory [62].

Ghrelin receptors are present throughout the GI tract including colon and rectum [17]. Studies in rat show that ghrelin stimulates motility in the small intestine [33, 34] and colon [97]. The effect of ghrelin on upper GI motility in humans was first studied in 2006 [98]. Infusion of ghrelin induced a premature gastric phase of the MMC in healthy volunteers and accelerated gastric emptying in patients with gastroparesis due to diabetes or surgical vagotomy [35, 36]. Supraphysiological doses of ghrelin or ghrelin agonists (ulimorelin) administered to healthy volunteers resulted in increased appetite and decreased satiety with few side effects [99]. A

correlation with elevated plasma glucose levels and free fatty acids was found [99]. In spite of these findings few studies have examined the effect of ghrelin on small bowel and colonic transit in human.

In our second study a 6 hours IV infusion of saline or ghrelin was given to healthy volunteers in order to measure gastric emptying, oro-caecal and colonic transit. Additionally, *in vitro* studies of human tissue from the GI tract were performed. As expected, our results demonstrated an increased gastric emptying rate and elevated hunger feelings during the ghrelin infusion, suggesting that the peptide was biologically active. We found no difference in oro-caecal or colonic transit time between the study groups and the number of bowel movements were unchanged. One of the recognised incretins, GLP-1, was significantly elevated but not GIP, PYY or leptin. With the increased rate of gastric emptying seen during an infusion of ghrelin there is a faster emptying of nutrients into the duodenum and subsequently a faster stimulation of the GLP-1 producing L-cells. The fact that PYY (co-localized in the same L-cells) remained unchanged was surprising, but may be due to the composition of the meal used.

Postprandially the plasma concentrations of glucose and insulin were significantly elevated as was the insulinogenic index calculated from the total period of 360 min. There was also a significant correlation between the release of insulin and glucose uptake. The relationship between release of GLP-1 and uptake of glucose was only borderline significant. The findings of elevated glucose concentrations in plasma are in line with other studies [53, 100, 101]. More recently, studies have suggested that exogenously given ghrelin directly stimulates the liver to increase glucose output by both enhanced glycogenolysis and gluconeogenesis [102]. The elevated glucose and insulin levels may reflect an impaired insulin sensitivity with decreased peripheral uptake of glucose. Ghrelin has been found to have no effect [99, 103], stimulatory [104] or inhibitory effect [100] on insulin secretion. During fed conditions the majority of studies show that ghrelin delays glucose stimulated insulin secretion by the pancreas [52, 105, 106]. In our study the elevated GLP-1 levels during the ghrelin infusion may contribute to the increased plasma concentrations of insulin and is likely due to the rapid entry on nutrients into the small intestine after the increased rate of gastric emptying.

Our results in this study do not support the hypothesis that an intravenous infusion of ghrelin stimulates small bowel and colon motility in healthy humans. The effect of ghrelin seems to be limited to the stomach and duodenum. Our study was conducted under fed conditions but are in line with a study by Tack *et al* where IV administration of ghrelin to fasted healthy volunteers induced a premature phase III fasted motor activity and increased the gastric tone [98]. Our findings are supported by our *in vitro* studies of tissues from different parts of the GI tract where only gastric tissue responded with enhanced contractile response to electrical field stimulation. Previous studies have confirmed that gastric motility is dependent of an intact vagus nerve [28, 107], although the local enteric nerve system may in some cases compensate for the lack of vagal input [34].

In our study the inability of ghrelin to stimulate colonic motility may be due to a poor penetration of the central nervous system. It is well known that the distal colon and rectum are innervated by the autonomic nerve system and that the defecation control centre is located in the lumbo-sacral spinal cord. Studies with synthetic ghrelin receptor agonists applied directly into the region cause propulsive contractions in rat [97], as well as, contractile activity of the

bladder [108]. Peripheral administration of ghrelin receptor agonists, able to cross the BBB, are more likely to stimulate the colon motility [109]. Recent studies have confirmed the presence of ghrelin receptors on preganglionic neurons in the spinal cord of mice [110] and that the ghrelin agonist, capromorelin, even stimulates defecation in rats after spinal cord injury [108].

One critique of our study may be the methods used for assessing the gastric emptying and transit time of small bowel and colon. The "golden standard" to assess gastric emptying is scintigraphy. The advantage of this test is that both solid and liquid meal may be studied and sometimes show the distribution within the stomach. The disadvantage is the high cost, the requirement of radioactive substances and a gamma camera. In our study the acetaminophen (paracetamol) tracer technique was used. This method is commonly used and correlates well with the scintigraphic technique, but is less expensive and does not require handling of radioactive material [90]. The lactulose breath test is the most widely used technique to assess oro-caecal transit in human. False positive results may be obtained if the small bowel contains bacteria. This was ruled out as the baseline level was low and no early rise was seen. Another disadvantage of the method is the lack of reference time in healthy individuals and "normal" oro-caecal time may vary between 1-6 hours depending of the food or fluid contents. The strength in this study was a cross-over study with the same individual studied twice in a similar way. Finally, the colon transit method in our study was modified from the commonly used method for assessing slow transit. To ensure that markers were located in the intestine and not the stomach, different markers were administered the evening prior to the study and the same morning. As the mean oro-caecal transit time in our study was 40 ±7.8 min in the ghrelin group, it would have been preferable to perform the x-rays before and directly after the infusion as well. However, the number of abdominal X-rays that we could perform were limited by the Radiation Protection Committee.

In summary, this study shows that supraphysiological doses of ghrelin fasten the gastric emptying but do not affect the oro-caecal or colon transit in healthy human volunteers. The 6-hour infusion of ghrelin resulted in postprandially elevated levels of glucose, insulin and GLP-1, most likely due to the enhanced gastric emptying rate.

The interesting finding that ghrelin stimulates gastric emptying in animal models of POI [30, 111, 112] encouraged us to further investigate the possibility of obtaining the same results in patients undergoing major abdominal surgery. POI is a common problem in surgery and may lead to symptoms as nausea, abdominal bloating and pain. Even though the ERAS concept (enhanced recovery after surgery) improves the clinical outcome, prolonged hospital stay due to POI is still common. We therefore performed a randomised double-blinded study where patients undergoing surgery for cancer or diverticulitis received a 3-hour infusion of ghrelin or saline before surgery and on post-operative day 2.

We found that an infusion of ghrelin two days after open bowel resection increases the gastric emptying rate and shortens the time to first defecation. The GLP-1 and GIP responses, as well as, PP and PYY were unchanged before and after the surgery in both study groups. In contrast, we found a significant pre-operative increase of insulin before surgery in the ghrelin group. The total amount of absorbed acetaminophen (measured as AUC) during saline infusion was significantly lower after surgery compared to before. The ghrelin infusion normalized the delayed emptying postoperatively. These results are in agreement with the

well-known fact that abdominal surgery causes paralysis of the upper GI tract. The findings that a ghrelin agonist enhance the gastric emptying rate after colectomy has previous been reported by Popescu et al [113]. However, the primary endpoint in their study was time to first bowel movement and secondary endpoint return of GI function. Patients receiving the ghrelin agonist experienced less nausea and vomiting which was interpreted as accelerated recovery of the upper GI tract. We did not observe any difference in nausea, hunger or fullness during the ghrelin infusion in our study. In this study observations on day 2, were chosen due to various reasons. Intake of oral fluids should be tolerated and pain management with epidural catheter established (avoidance of opioids). Additionally, confounding factors related to the anaesthesia should be excluded as well as normalizing of stress-induced elevation of endogenous levels of ghrelin [114].

The finding that a 3-hour infusion of ghrelin shortens time to first defecation indicates that ghrelin modulates the total GI transit in patients undergoing bowel surgery. The patients were allowed intake of solid food already on day 1 after surgery. Therefore, nutrients had been introduced into the intestine before the infusion of ghrelin enabling bowel movements. As discussed above, the same results in human have been obtained with administration of the ghrelin agonist [113]. Studies in rat have shown that intracerebral administration of ghrelin decreased the colonic transit time up to 43% [115]. The same results with stimulated propulsive activity in colon were seen with injected ghrelin into the spinal cord [116]. It seems unlikely that ghrelin in our study was able to pass the BBB and stimulate the central nervous system. The most likely explanation for our finding is that the hastened gastric emptying postoperatively facilitates bowel movements via the gastro-colic reflex. In contrast to our second study we found no elevation of GLP-1 or other GI peptides that influence motility despite the stimulated gastric emptying. This might be due to the different composition of the meal and the post-operative metabolic condition. An unchanged GLP-1 response to colon surgery has also been shown in a study by Palnaes Hansen [117] and Nauck et al [118].

In line with our second study, the infusion of ghrelin increased the plasma concentrations of glucose. The mechanism behind the significant increase of insulin before surgery is not clear. In normal conditions ghrelin has been shown to inhibit insulin secretion. After surgery, in spite of the ERAS concept, patients show a decreased insulin sensitivity [119], which could result in elevated levels of insulin. We suggest that the role of ghrelin in glucose homeostasis after intestinal surgery is further studied. Our study has some weaknesses. The number of study subjects is small and the colorectal surgical procedures vary between the groups. There was no significant difference in the time it took to complete the surgery or blood loss, but different parts of colon and distal ileum were resected. Three patients in each study group received a stoma (one ileostomy and two sigmoidal stomas, respectively) which could also affect the results. Furthermore not all patients were studied both before and after surgery due to complications of the surgery and mistaken administration of acetaminophen on the study day.

In summary, this study shows that a 3-hour infusion of ghrelin can increase the rate of gastric emptying in patients with POI. We also found that time to first defecation was shorter with little effect on GI peptides.

To investigate the role of endogenous ghrelin, studies in rodents have been done with knockout mice and with receptor antagonists injected peripherally or intracerebrally. In humans no studies of ghrelin antagonists have been performed. After RYGB surgery for obesity it has been reported that plasma concentrations of ghrelin were both decreased [49] and increased [120]. Thus, it is possible that effects of endogenous ghrelin could be studied after RYGB surgery. In contrast, other GI peptides such as GLP-1 have been shown to be elevated.

In our fourth study, we investigated alterations in ghrelin and other GI peptides, as well as, glucose responses early after RYGB surgery for obesity. Further the rate of gastric (and pouch) emptying was studied. Our hypothesis to use RYGB as model to study endogenous ghrelin was not proven correct as plasma concentrations of both fasting and postprandial ghrelin were unchanged with the exception of on day 3. Changes in plasma concentrations of ghrelin are unlikely to contribute to the improved metabolic control seen after RYGB surgery. There have been conflicting reports with regard to alterations of ghrelin after RYGB as noted above [121]. This has been attributed to different surgical techniques and to which extent the vagus nerve is affected by the surgery. Fasting GLP-1 levels were unchanged as well, but increased postprandially during the year. Fasting levels of GIP were significantly decreased on day 3 and 1 year after surgery but $AUCs_{0\text{-}180}$ were unchanged before and after surgery.

In line with previous studies we found gradually lower values of fasting blood sugar and insulin levels during the first year. This was also confirmed with the HOMA test where the index decreased from over 6 to normal values below 2.8. The mechanism behind the early amelioration of the glucose homeostasis (day 3) is not clear but may be explained by energy restriction and increased postprandial levels of GLP-1. The hunger feelings, assessed with the VAS scale, were significantly decreased already day 3 as well as feelings of fullness were high, which also may be attributed to the elevated postprandial GLP-1 levels. Fasting insulin improved, reflecting the weight loss and improved insulin sensitivity [122].

The rapid rise of glucose (and incretin hormones) is likely due to the rapid entry of nutrients into the small intestine after RYGB. We found a rapid pouch emptying after the surgery. Again, this was not due to alterations in ghrelin levels.

In summary, our study of RYGB surgery shows a rapid emptying of the upper pouch. This results in increased levels of GLP-1 after meal, which may contribute to the improvement in glucose homeostasis seen early after the procedure. Ghrelin does not contribute to this, as plasma concentrations of ghrelin were essentially unchanged.

In conclusion, this thesis shows that exogenously administered ghrelin increases the rate of gastric emptying in humans, both in healthy volunteers and after major abdominal surgery. The sister hormone obestatin seems to have little effect on GI motility. In our hands plasma concentrations of ghrelin are unchanged after RYGB for obesity, yet the rapid entry of nutrients into the small intestine results in increased plasma concentrations of glucose and GLP-1. This highlights the importance of the stomach in regulating glucose metabolism. A rapid entry of nutrients into the small intestine, regardless if it is due to a faster gastric emptying after the administration of ghrelin or bypass of the stomach in bariatric surgery, results in elevated plasma glucose and GLP-1. Normally the pylorus regulates the amount of nutrients that enter the duodenum. In the setting of our studies, the increased rate of gastric

(pouch) emptying after ghrelin infusion or RYGB, GLP-1 seems to be a central player that tries to counteract the rapid entry of nutrients into the intestine by slowing gastric emptying and increasing plasma insulin to balance glucose metabolism.

It is possible that the physiological role of ghrelin is to prepare the GI tract for a meal and initiate gastric emptying, which is then counteracted by the release of incretins, which serve to inhibit gastric emptying and propulsion through the small bowel, as well as promote insulin release in order to balance a rapid nutrient uptake. It is important to realise that ghrelin acts in concert with a range of other GI peptides that are released separately and act differently to achieve metabolic control.

12.0 CONCLUSIONS

- Obestatin does not seem to play a role in the modulation of GI gastric motility.
- In healthy humans effect of ghrelin on GI motility is limited to the stomach and does not affect small bowel or colonic motility.
- After open colo-rectal surgery ghrelin accelerates the rate of gastric emptying and time to first bowel movement.
- Plasma concentrations of ghrelin are unchanged after RYGB which makes it unlikely that ghrelin contributes to the metabolic improvements seen after the surgery.
- The rate of pouch emptying after RYGB is very rapid which likely contributes to the rapid rise of glucose, insulin and GI peptides seen after the surgery.

13.0 POPULÄRVETENSKAPLIG SAMMANSTÄLLNING

Idag finns ett 30-tal kända peptider eller hormoner i mag-tarmkanalen som reglerar födointag, aptit, energi och sockeromsättning. Dessa peptider signalerar till olika centra i hjärnan. Peptiderna ghrelin och obestatin, som upptäcktes 1999 respektive 2005, härrör från samma ursprung men stimulerar olika receptorer. Ghrelinreceptorn finns i ett stort antal vävnader såsom mag-tarmkanalen, bukspottskörteln, njurar, fettvävnad, skelettmuskel och hjärna. Det finns två huvudsakliga molekylära former av ghrelin, acyl och des-acyl ghrelin med motsatta effekter på en rad funktioner i mag-tarmkanalen. Mag-tarmkanalens motorik styrs förutom av peptidhormoner även av det lokala nervsystemet i tarmen och nerver från ryggmärg och hjärna. Peptidhormonerna kan verka lokalt i tarmen, transporteras med blodet till hjärnan eller produceras direkt i hjärnvävnaden. Ghrelin påverkar en rad olika funktioner och styr hunger och måltidsreglering, sockeromsättning och fettförbränning. Obestatins roll i samspelet med ghrelin är oklar.

Syftet med de studier som presenteras i denna avhandling var att undersöka hur ghrelin påverkar mag-tarmkanalens motorik samt ämnesomsättning hos råtta och människa.

I studie 1 studerade vi förmågan hos obestatin och ghrelin (enskilt eller i kombination) att påverka enskilda muskelfibrers kontraktionsförmåga samt råttans tarmrörelsemönster och magsäckstömning. Vi fann att obestatin inte stimulerar motoriken och endast påverkar ghrelinet svagt. Ghrelin ger däremot en ökad magsäckstömning och stimulerar fastemotorik med hungersugningar.

I studie nr 2 erhöll friska försökspersoner vid två tillfällen ghrelin eller saltlösning under 6 timmar i följd för att jämföra effekten på magsäckstömning samt rörelsemönster i tunntarm och grovtarm. Frisättningen av flera mag-tarmhormoner och glukosomsättningen undersöktes. Vi fann en ökad magsäckstömning men ingen påverkan på passagetid i tunntarm eller grovtarm. Glukos- och insulinnivåer samt peptiden GLP-1 steg efter ghrelin.

I studie 3 deltog patienter som genomgick kolo-rektal kirurgi på grund av tarmcancer eller tarmfickor. Preoperativt och två dagar postoperativt gavs ghrelin eller saltlösning under 3 timmar. Magsäckstömning samt tid till första tarmtömning mättes liksom förändringar av tarmhormoner i blodet. Ghrelin gav snabbare magsäckstömning och kortare tid till första tarmtömning. Vi fann även förhöjda värden av insulin pga snabb magsäckstömning.

Det primära syftet med studie 4 var att belysa de tidiga hormonella följderna av överviktskirurgi med gastric bypass kirurgi (RYGB). Kroppsegna nivåer av ghrelin och insulin samt peptiderna GIP och GLP-1 mättes och sockeromsättning studerades. Vi fann dubblerad hastighet av magsäckstömningen och förbättrad sockerbalans. Nivåerna av GLP-1 var samtidigt förhöjda.

Sammantaget visar arbetena att ghrelin har en kraftigt stimulerande effekt på hunger och magsäckens motorik och ger förkortad postoperativ hämning av tarmmotoriken. Detta ger sekundära effekter på ämnesomsättning och metabol kontroll genom att frisättningen av flera peptidhormoner från mag-tarmkanalen stimuleras. Överviktskirurgi med RYGB som också ger en mycket snabb tömning av näring till tarmen ger likartad hormonfrisättning, ff a av GLP-1, vilket ger mättnad och förbättrad metabol kontroll. Vi fann däremot inte att obestatin, föreslagen som motverkande till ghrelin, hade några effekter på mag-tarmkanalens motorik.

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16.0 APPENDIX

Study II

Questionnaire of bowel habits

DAG 0 (dagen innan ditt första besök)

Besvara följande frågor VARJE GÅNG du har tarmrörelser

Tid på dagen:	_ _:_ _	_ _:_ _	_ _:_ _	_ _:_ _	_ _:_ _
1. Hade du gasavgång?	**Ja Nej**	**Ja Nej**	**Ja Nej**	**Ja Nej**	**Ja Nej**
2. Hade du avföring?	**Ja Nej**	**Ja Nej**	**Ja Nej**	**Ja Nej**	**Ja Nej**
3. Kände du att du kunde tömma tarmen fullständigt?	**Ja Nej**	**Ja Nej**	**Ja Nej**	**Ja Nej**	**Ja Nej**

4.Var buken uppblåst eller utspänd? **Ja Nej**

5. Kände du smärtor eller obehag i buken? **Ja Nej**

Kommentar:……

Study II

Study protocol DAY 1

DATE

Date of visit: ☐☐☐☐☐☐☐☐ (D D M M M Y Y Y Y) Arrival time: ☐☐:☐☐

SITZMARKS CAPSULE

Has the subject taken the second Sitzmarks capsule?

Yes ☐ Time: ☐☐:☐☐

No ☐ please specify: _____

X-RAY

X-ray performed in the morning?

Yes ☐ Time: ☐☐:☐☐

No ☐ please specify: _____

FASTING PLASMA SAMPLE AND EXHALED HYDROGEN

Fasting plasma sample taken?

Yes ☐ Time: ☐☐:☐☐

No ☐ please specify: _____

GHRELIN/SALINE INFUSION

Has the infusion been given?

Yes ☐ start: ☐☐:☐☐ stop: ☐☐:☐☐

No ☐ please specify: _____

ENERGY-FIXED BREAKFAST (450 kcal, _____ grams)

Rejected breakfast: ☐☐☐ grams. Breakfast time: ☐☐:☐☐

Study protocol DAY 1

SUBSTANCE INTAKE

Has the following substances been ingested during breakfast?

1.5 g of paracetamol (Alvedon)

Yes ☐

No ☐ please specify: _____

15 ml of lactulose

Yes ☐

No ☐ please specify: _____

ENERGY-FIXED LUNCH (600 kcal, _____ grams)

Rejected lunch: ☐☐☐ grams. Lunch time: ☐☐:☐☐

PLASMA SAMPLING

Minutes:	Actual time:	Signature:	Hours:	Actual time:	Signature:
15	☐☐:☐☐	_____	4	☐☐:☐☐	_____
30	☐☐:☐☐	_____	5	☐☐:☐☐	_____
45	☐☐:☐☐	_____	6	☐☐:☐☐	_____
60	☐☐:☐☐	_____			
90	☐☐:☐☐	_____			
120	☐☐:☐☐	_____			
180	☐☐:☐☐	_____			

Study protocol DAY 1

MEASUREMENTS OF EXHALED HYDROGEN

Minutes:	Actual time:	Measurement:	Minutes:	Actual time:	Measurement:
10	__:__	_____ ppm	100	__:__	_____ ppm
20	__:__	_____ ppm	110	__:__	_____ ppm
30	__:__	_____ ppm	120	__:__	_____ ppm
40	__:__	_____ ppm	130	__:__	_____ ppm
50	__:__	_____ ppm	140	__:__	_____ ppm
60	__:__	_____ ppm	150	__:__	_____ ppm
70	__:__	_____ ppm	160	__:__	_____ ppm
80	__:__	_____ ppm	170	__:__	_____ ppm
90	__:__	_____ ppm	180	__:__	_____ ppm

VISUAL ANALOG SCALE (VAS)

Time:	Actual time:	Hunger	Fullness	Nausea	Signature
08:45 **infusion**	__:__				
09:15	__:__				
10:00	__:__				
11:00	__:__				
12:00	__:__				
	__:__				
	__:__				
	__:__				
	__:__				

I confirm that all data captured at this visit are correct and completed to the best of my knowledge

Investigator's signature: _____ Date: _____

D D M M M Y Y Y Y

DAY 2

DATE

Date of visit: |_|_|_|_|_|_|_|_|_|_| Arrival time: |_|_|:|_|_|
D D M M M Y Y Y Y

X-RAY

X-ray of abdomen performed?

Yes ☐ Time: |_|_|:|_|_|

No ☐ please specify: _____

I confirm that all data captured at this visit are correct and completed to the best of my knowledge

Investigator's signature: _____ Date: |_|_|_|_|_|_|_|_|_|_|
D D M M M Y Y Y Y

DAY 3

DATE

Date of visit: |_|_|_|_|_|_|_|_|_|_| Arrival time: |_|_|:|_|_|
D D M M M Y Y Y Y

X-RAY

X-ray of abdomen performed?

Yes ☐ Time: |_|_|:|_|_|

No ☐ please specify: _____

BOWEL HABITS QUESTIONNAIRE

Has the subject completed the bowel habits questionnaire?

Yes ☐

No ☐ please specify: _____

I confirm that all data captured at this visit are correct and completed to the best of my knowledge

Investigator's signature: _____ Date: |_|_|_|_|_|_|_|_|_|_|
D D M M M Y Y Y Y

Visual analogue scale

Fyll i detta formulär klockan

Markera med ett streck på varje linje hur Du upplever hunger, mättnad och så vidare just nu!

1 Hur hungrig känner Du Dig just nu?

Inte alls hungrig _____ **Mycket, mycket hungrig**

2 Hur mätt är Du just nu?

Inte alls mätt _____ **Mycket, mycket mätt**

Hur mycket illamående upplever Du just nu?

Inget illamående alls _____ **Maximalt illamående**

Namn _____

Datum _____